**REMARKS**

Claims 1, 2 and 5-17 are pending in the instant application. Claims 1, 2 and 5-17 have been rejected. Claims 1, 2, 8-10, and 16-17 have been amended. Claims 6 and 7 have been canceled. No new matter has been added by these amendments. Reconsideration is respectfully requested in light of the following remarks.

**I. Priority Under 35 U.S.C. §119(e)**

Priority to U.S. provisional patent application Serial No. 60/090,849 has not been granted for claims 6, 7, 9, 10, 16 and 17 as the Examiner suggests that the provisional application does not provide support for enhancing or suppressing at least the humoral immune response or CD4 Th1 immune response to a target antigen in an "aged or immuno-compromised individual" (claim 6); a target antigen in a "human subject fifty years or older" (claim 7); the use of a "toxin" as an antigen (claim 9); treating a cancer or tumor cell of the "lung, head and neck, uterine and leukemia" (claim 10); treating a "protozoan disease" (claim 16); treating "leishmaniniasis, Listeriiosis, leprosy, or tuberculosis infection" (claim 17) as recited in the claims. The Examiner suggests that the filing date of said claims is the filing date of the priority application PCT/US99/12825.

Applicants respectfully disagree, however, in an effort to facilitate the prosecution of the instant application, Applicants have removed reference to the above subject matter by canceling claims 6-8 and amending claims 9-10 and 16-17. Withdrawal of this objection is therefore respectfully requested.

**II. Objection of Claims Under 35 U.S.C. §132**

The amendment filed 5/24/04 has been objected to under 35 U.S.C. §132 for introducing new matter into the disclosure. The Examiner indicates that the original disclosure does not support the recitation of "and gp72" in the paragraph bridging pages 10 and 11. Applicants have corrected this inadvertent typographical error. Withdrawal of this objection is therefore respectfully requested.

**III. Rejection of Claims Under 35 U.S.C. §112**

Claims 1, 2 and 5-17 have been rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Specifically, the Examiner suggests that *in vitro* and animal model studies have not correlated well with *in vivo* clinical trial results in patients. It is suggested that it is not clear that the reliance on experimental observations accurately reflects the relative efficacy of enhancing or suppressing the humoral immune response or CD4 Th1 immune response to a target antigen comprising any selected antigen and anti-CD40 antibody. The Examiner suggests that pharmaceutical therapies, absent clinical data, are unpredictable as the protein may be inactivated before producing an effect, the protein may not reach the target area, and other function properties may make the protein unsuitable for *in vivo* therapeutic use. The Examiner concludes that in view of the lack of predictability of the art, undue experimentation would be required to practice the claimed methods with a reasonable

expectation of success. Applicants respectfully traverse this rejection.

MPEP 2164.02 states that an *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. The use of rodents to establish therapeutic efficacy in humans is well-documented in the art. To illustrate, Delmonico and Cosimi ((1996) *Clin. Transplant.* 10(5):397-403), in view of Gorczynski et al. ((1995) *Transpl. Immunol.* 3:321-9), teach that the immunomodulatory activity of an anti-CD4 monoclonal antibody in rodent models correlates well with human clinical trials. Therefore, as the specification teaches that mice receiving an antigen-anti MHCII monoclonal antibody in combination with an anti-CD40 antibody results in significantly enhanced yields of IgG antibody to the antigen as compared to mice receiving antigen alone, one of skill in the art would consider this a reasonable working example of the method of the present invention.

MPEP 2164 further indicates that the amount of guidance or direction needed to enable the invention is inversely related to the amount of knowledge in the state of the art as well as the predictability in the art. In re Fisher, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970).

Delmonico and Cosimi also teach that the state and level of skill in the art of immunomodulation at the time of filing was quite developed as evidenced by the progressive improvement in clinical outcome upon changes in the structure of the anti-CD4 antibody. Furthermore, this reference demonstrates that the delivery of intact antibodies (and likewise antibody conjugates)

is predictable and would provide a reasonable expectation of successfully effecting an immune response.

Thus, in view of the teachings of the prior art and the high level of skill in the art for modulating the immune response, the present application provides sufficient guidance in its description of *in vivo* and *in vitro* therapeutic efficacy for one of skill to use a conjugate comprising a selected antigen and an antibody that specifically binds to a molecule which is expressed by an APC; and an anti-CD40 antibody in methods of enhancing or suppressing the humoral immune response or CD4 Th1 immune response to the target antigen. Withdrawal of this rejection is therefore respectfully requested.

Claims 1, 2 and 5-7 have further been rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. It is suggested that the recitation of "thereby synergistically enhancing or suppressing at least the humoral immune response or CD4 Th1 immune response to the target antigen" renders the claims indefinite because this phrase is relative in nature. The Examiner suggests that the specification as filed provides no support for "synergistic enhancement" as the combination of an antigen-antibody conjugate and an anti-CD40 antibody acting together to have an effect which is greater than the simple sum of their effects when acting alone. Applicants respectfully disagree.

In an effort to clarify the teachings of the instant disclosure and Examples, claim 1 has been amended to remove reference to a synergistic effect and recite that the use of an

antigen-antibody conjugate in combination with an anti-CD40 antibody enhances or suppresses the immune response as compared to the activity of the antigen-antibody conjugate in the absence of the anti-CD40 antibody. Withdrawal of this rejection is therefore respectfully requested.

**IV. Rejection of Claims Under 35 U.S.C. §103**

Claims 1, 2, and 5-17 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Anand et al. (U.S. Patent No. 6,291,208) and Heath (U.S. Patent Application No. 2002/0135722) and further view of Applicants' admission that species of classes and types of antigens are held obvious in view of one another in the instant invention.

The Examiner suggests that Anand et al. teach the use of antibody conjugates comprising antibodies that bind antigen presenting cells, including dentritic cells, to deliver antigens in order to generate immunogenic compositions to a variety of antigens and that this is applicable to any antigen derived from viruses, bacteria and tumors.

It is further suggested that Heath teaches the co-administration of a CD40 stimulating moiety (*e.g.*, anti-CD40 antibodies) and the appropriate antigen, including the use of covalent linkage or co-entrapment as a vaccine to a variety of antigens.

Thus, the Examiner suggests that given the teachings of Heath to provide anti-CD40 with antigen in composition form or as a conjugate and the teachings of Anand et al. to provide antigen with anti-antigen present cell/dendritic cell antibodies, it would have been obvious to one of ordinary skill in the art to

administer the antigen in the context of such antigen-antibody conjugate with the immunostimulatory anti-CD40 antibodies to boost the immune response to a wide variety of desired antigens, including providing both components in the same composition, as taught by Heath. It is suggested that the motivation to combine the prior art can arise from the expectation that the prior art elements will perform their expected function to achieve their expected results when combined for the common known purpose.

Applicants respectfully traverse this rejection. The cited references fail to teach, suggest, or motivate one of skill in the art to combine the three components with the associations recited in the instant claims. Anand et al. teach targeting an antigen to an antigen-presenting cell using an antibody; however, this reference does not teach or suggest the use of an anti-CD40 antibody. Likewise, Applicants find no reference to, or a suggestion of, the use of an antibody to target the antigen to an antigen-presenting cell in the Heath disclosure. In fact, based upon the teachings of Heath, one of skill would not be motivated to attach the antigen to anything other than an anti-CD40 antibody because the coupling of anti-CD40 to antigen disconnects the adjuvant effect from the toxicity of anti-CD40. Heath teaches that attaching the CD40 antibody to antigen results in no toxicity, "while the adjuvant effect remains strong, in fact stronger than that of the mixture." See paragraph 0132 of Heath. Likewise, Anand et al. teach that the use of adjuvants can be avoided by using an antigen-antibody conjugate of the disclosure.

A prior art reference must be considered in its entirety, *i.e.*, as a whole, including portions that would lead away from the claimed invention. W.L. Gore & Associates, Inc. v. Garlock,

Inc., 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984). MPEP 2141.02. Thus, one of skill would have little motivation for combining the teachings of Anand et al. with those Heath, because both references teach away from such a combination. Therefore, it is respectfully requested that this rejection be withdrawn.

## V. Conclusion

The Applicants believe that the foregoing comprises a full and complete response to the Advisory Action of record. Accordingly, favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,

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# Anti-CD4 monoclonal antibody therapy

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Abstract: Because they can be selected to target only cells which are crucial for rejection, monoclonal antibodies (mAbs) offer enormous potential for specific manipulation of the immune response. Interest in the clinical potential of anti-CD4 mAbs has been heightened by the demonstration, in experimental models, that such therapy can produce long-term donor-specific non-responsiveness. Early clinical trials using two murine anti-CD4 mAbs (BL4 and mT-151) were discouraging, with over 50% of recipients suffering early rejection episodes. Another murine preparation, OKT4A, was initially found to prolong allograft survival in non-human primates. Limited clinical trials revealed that this mAb was well tolerated, that most recipients produced an anti-murine response, and that only 26% of patients suffered rejection episodes during the first 3 post-operative months. Another murine preparation, Max.16H5, has been reported to reverse late onset acute rejection episodes as effectively as, but more safely than, conventional immunosuppression. More recent interest has focused upon humanized recombinants of these earlier murine anti-CD4 preparations. cMT-412, has been studied in recipients of heart or heart–lung allografts. These patients were observed to have less frequent and markedly delayed rejection episodes, fewer infectious complications, and better overall survival than that observed in an ATG-treated control group. Further studies are thus being undertaken. A CDR grafted IgG4 preparation of OKT4A has also been studied. This molecule (OKTcdr4a) contains only 8% of the parent murine sequence while retaining the binding affinity of OKT4A for the human CD4 antigen. In a pilot trial, biopsy-proven reversible rejection episodes were observed in 2/11 (18%) of renal allograft recipients. There were no allograft failures and no antibody response to the mAb. These and other trials emphasize the intense interest in immunosuppressive regimens incorporating anti-CD4 mAbs as well as the difficulties encountered in defining optimal protocols. Nevertheless, the impressive results observed in rodent and non-human primate models suggest that these agents are likely to play an important role in future immunosuppressive protocols, particularly those designed to induce tolerance.

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Key words: monoclonal antibodies – anti-CD4 – tolerance

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The concept of selective T-cell immunosuppression was initially investigated at the Massachusetts General Hospital, shortly after the identification of the two major T-cell subsets characterized by exclusive CD4 and CD8 antigens (1, 2). The rationale for the study of a specific anti-CD4 immunosuppressive approach was based upon the hypothesis that CD4 T-cell sensitization was central to the immune response, providing the necessary immunologic signals for CD4 T-cell proliferation and for CD8 T-cell recruitment and effector activities. Functional attributes of the T-cell subsets had been determined in vitro, and these characteristics became the terminology applied to each subset. The CD4 subset was originally referred to as the "helper/inducer" population, and the CD8 T-cells were termed "cytotoxic / suppressor".

Although CD4 and CD8 T-cell populations are seldom identified with such reference today, most of the original concepts regarding the functional capacity of each subset have been verified by more than a decade of study. The vigor of the immune re-

sponse is clearly dependent upon CD4 T-cells (3). This response is initiated by the presentation of MHC Class II alloantigen, specifically to the CD4 T-cell. The amplification of the rejection process is accomplished by cytokines, released again specifically from the CD4 T-cell. Thus, the impetus for a stepwise strategy of investigation, which would selectively disable the CD4 T-cell, at first in non-human primates and then in human allograft recipients, was derived from the inherent aim of immunosuppression: to prevent rejection.

Monoclonal antibodies to CD4 T-cells were obtained for our studies from a panel of mAbs produced by Kung and colleagues of the Ortho Pharmaceutical Company, utilizing the hybridoma technique of Kohler and Milstein (4). Although these mAbs were raised in mice against human lymphocytes, many of them were found to be cross-reactive with the lymphocytes of non-human primates. This fortuitous cross-reactivity provided the opportunity for in-vivo efficacy and toxicity screening. This technology also provided a unique opportunity to clarify the immunopathologic mechanisms involved in the allograft response because of the specificity of antilymphocytc antibodies to exclusive T-cell targets.

### Pre-clinical studies: murine OKT4 and OKT4A

Murine OKT4 mAb treatment was first evaluated in Cynomolgus renal allograft recipients in 1980 (5). Seven allograft recipients were given OKT4 as the only immunosuppressive agent administered intravenously 2 d prior to the renal allograft procedure and continued on a daily or alternate day basis, for 7–14 d. A dose of 0.5–1.0 mg/kg was sufficient to achieve serum excess for 24–48 h following each OKT4 administration. Allograft survival in 6 evaluable animals was extended to as long as 42 d, whereas control animals developed terminal rejection between 8 and 11 d following transplantation. Immunologic monitoring of peripheral blood lymphocytes utilizing a panel of fluoresceinated mAbs and flow cytometry revealed gradual but incomplete clearance of CD4+ T-cells. The number of reactive cells in the peripheral blood fell from pre-treatment levels of 809±130/mm$^3$ to a range of 200–300/mm$^3$ by the third to fifth day of therapy.

As part of these initial studies, a systematic investigation of the epitopic structure and possible polymorphism of the CD4 molecule was also undertaken (6). Multiple epitopes were identified, and population studies showed that some human subjects completely lacked the OKT4 reactive site. Subsequent therapeutic trials have, therefore, used OKT4A, a murine IgG2a anti-CD4 immunoglobulin which binds to a universally expressed epitope of the human CD4 antigen that is separate from and non-competing with the epitope recognized by OKT4.

Cynomolgus renal allograft recipients have been treated with several dose schedules of murine OKT4A (7, 8). These have ranged from 0.1–0.3 mg/kg/d, given for 12 d, to a single dose of 3.6–10 mg/kg administered only on the day of transplantation. The maximum allograft survival for control animals who received either no therapy, F(ab')2 fragments, or a mAb non-reactive with Cynomolgus T-cells (OKT3) was 11 d. Prolonged allograft survival was achieved with each of the OKT4A protocols. The longest allograft survival (mean of 39±6.4 d) was observed in recipients of the single high-dose of 10 mg/kg.

### Pre-clinical studies: humanized OKT4A

The encouraging results with these murine mAbs stimulated further investigation of OKT4A immunosuppression using a progressively humanized molecule (9). The objectives of humanizing the murine OKT4A molecule were to reduce the immunogenicity of the xenogeneic agent and to provide a reagent that would hopefully be more effective in recruiting appropriate immunomodulating responses in human allograft recipients.

The complementary determining regions (CDR) of murine OKT4A were grafted onto a human antibody variable region framework as previously described (10). The resultant structure was then combined with either a human IgG1 or IgG4 constant region. These IgG1 and IgG4 constant region isotypes were chosen because of their differential binding of complement, and thus for their potential to produce either T-cell depletion via complement receptors (IgG1 only) or coating and/or modulation of the CD4 antigen (IgG4) without T-cell destruction. The final OKTcdr4a mAbs which were produced (both IgG1 and IgG4) contained 8% of the murine OKT4A sequence, and yet they retained comparable binding affinity for the CD4 antigen to that of the parent murine OKT4A.

Multiple low-dosage (1mg/kg/d ×12) and single high-dose (10 mg/kg) protocols of both the IgG1 and the IgG4 OKT4A mAb were administered to Cynomolgus renal allograft recipients. Allograft survivals comparable to those achieved with the purely murine OKT4A were observed (9). The longest survival (45±6.0 d) was again noted following administration of the single high-dose regimen.

### Clinical trials: murine OKT4A

As these non-human primate investigations were defining the potential usefulness of humanized

OKT4A, the encouraging observations from the previous monkey studies of the murine mAb had already justified a pilot trial of that preparation in human cadaver donor (CD) renal allograft recipients. This study was conducted by the NIAID/NIH sponsored Cooperative Clinical Trials in Transplantation (CCTT) (11). It was designed to administer murine OKT4A for 12 consecutive days, beginning within 3 h of the transplant procedure. A standard regimen of cyclosporine (CsA), azathioprine and prednisone was also administered. The primary objectives of the trial were to evaluate OKT4A tolerability and dose efficacy, CD4 T-cell immunomonitoring, episodes of rejection, and allograft survival.

A total of 30 patients were enrolled into the trial between August 1992 and October 1993 from three institutions: the University of Chicago, the University of Minnesota, and the Massachusetts General Hospital. Diabetic nephropathy, hypertension/nephrosclerosis, and interstitial nephritis/pyelonephritis were the causes of renal failure in 64% of the patients. Half of the patients had peak panel reactive antibody levels of greater than 10%. The average ABDR match was 1.8, with a maximum of 5 and a minimum of 0.

Patients were treated with dosages of 0.5 (24), 1.0 (3), or 2.0 (3) mg/kg/d. Patients with delayed graft function were to be excluded from study. Thus, recipients remained eligible for 12 consecutive days of OKT4A treatment if their serum creatinine at 24 h following transplantation was less than 85% of the pretreatment value. Otherwise, OKT4A was discontinued after the first dose. Recipients who received more than one dose of OKT4A, were deemed evaluable for outcome. OKT4A could also be discontinued if an episode of acute rejection was diagnosed prior to the conclusion of the planned OKT4A therapeutic course.

Nineteen of the 24 patients who were treated with the 05 mg/kg/d dosage were evaluable. Five patients were excluded because of delayed graft function (4), or donor age violation of protocol eligibility (1). Seventeen of the 19 patients received the 12-d course of treatment. Seven of the 19 patients (37%) were treated for presumed rejection within the first 3 months. Biopsy findings of rejection were mild, revealing mainly tubulitis. Endarteritis was evident in only 2 biopsies. Three patients experienced a second episode of rejection; however, there have been no allograft failures due to rejection among the 19 evaluable patients after nearly 2 yr of follow-up.

Five patients were evaluable at the higher dose levels of 1.0 and 2.0 mg/kg/d; 1 patient was excluded because of delayed graft function. An episode of rejection within 5 d following transplantation was conically diagnosed in 4 evaluable patients prompting discontinuation of OKT4A treatment. CsA levels were below the target range of 275–350 ug/ml (by TDX) at the time rejection was diagnosed in all 4 patients The diagnosis of rejection was not confirmed by allograft biopsy in 1 patient, and it was not corroborated by an independent review of the pathology specimen in another patient. Thus, definitive conclusions regarding the efficacy of the 1.0 and 2.0 mg/kg doses could not be established.

Since there was considerable variability in the rejection experience reported by the three study centers, a detailed review of individual clinical records was performed. This revealed major center differences in CsA blood levels during the OKT4A therapy. Episodes of rejection within the first 2 wk following transplantation were usually associated with CsA levels below the targeted therapeutic range. In this non-controlled study, however, it was not possible to conclude whether OKT4A provided additional protection in the presence of therapeutic CsA levels. In addition, not all patients who were clinically suspected of having acute rejection underwent a confirmatory allograft biopsy, and 30% (3/10) of the evaluable patients treated for rejection following allograft biopsy did not have the diagnosis of rejection confirmed by an independent panel of CCTT pathologists. Following this blinded review of the pathology, the confirmed 3-month rejection rate for the 19 evaluable 0.5 mg/kg patients was 26%.

In our own Unit, 8 patients were treated with the 0.5 mg/kg/d dose of murine OKT4A. Two patients experienced an episode of rejection. One of these patients died later of a myocardial infarction with a normally functioning allograft. The other 7 patients continue to do well, with creatinine levels of less than 2.0 mg %, 21–28 months following transplantation.

The murine OKT4A administered in this trial was well tolerated, without first-dose side effects. Adverse effects were generally mild and not specifically attributable to OKT4A therapy. Saturation of the T-cell CD4 epitope was achieved by the 0.5 mg/kg dose. Mean trough level saturations of the CD4 antigen of greater than 90% were achieved in 73% of all patients and 85% of the patients treated with a full course of OKT4A at the 0.5 mg/kg dose.

A HAMA (human anti-mouse antibody) response to the murine mAb was detected in 82% (18/22) of patients tested. In patients treated with a full course of OKT4A the median time to peak HAMA was 15.5 d; HAMA was detected, however, as early as 7 d following the initiation of OKT4A treatment. No association between HAMA response and the occurrence of rejection was observed.

The actuarial allograft survival rate at 2 yr following transplantation, for the 24 evaluable recipients of OKT4A (19 at the 0.5 mg/kg and 5 at the 1.0 and 2.0 mg/kg doses), was 83%. Eighteen of the 24 patients have completed their 2-yr follow-up clinic visit. There have been 4 allograft failures; 2 of these occurred in patients who died with functioning allografts. There have been no failures because of rejection.

The low rate of allograft failure, the minimal side effects, and the observation of a 3-month biopsy-confirmed rejection incidence of 26% in patients treated with the 0.5 mg/kg dose of OKT4A supported the continued investigation of an anti-CD4 approach to immunosuppressive therapy.

### Clinical trial: CDR-grafted OKT4A

The next phase of study was to administer a mAb mainly human in construction, except for the retained murine portion reactive to the CD4 antigen. The IgG4 CDR grafted OKT4A, which had been found in non-human primate renal allograft recipients to be as effective as the murine preparation, was chosen for study. The risk of potentially prolonged CD4+ T-cell depletion following IgG1 OKTcdr4a therapy was considered to be a possible hazard to human recipients. Significant CD4+ T-cell depletion was observed in Cynomolgus recipients of only the IgG1 CDR-grafted OKT4A (in some instances for 60–90 d following transplantation), in contrast to the coating/modulation (with minimal depletion) noted following the IgG4 OKTcdr4a treatment (9). Our decision to use the non-depleting preparation was also influenced by reports of other CD4-T cell depleting mAbs such as cMT-412 (12) which, when administered to human allograft recipients, had resulted in depressed CD4 T-cell levels for at least a month and in some instances much longer.

The necessity of achieving T-cell depletion to provide effective immunosuppression via mAb therapy remains controversial. Anti-CD4 mAbs could prevent CD4+ T-cells from responding to a foreign peptide (bound to MHC molecules on an antigen presenting cell), by modulating the CD4 antigen from the cell surface, by competitively coating the CD4 antigen, or by actually depleting the CD4+ T-cell population. All murine OKT4A treated non-human primates showed "coating" and/or modulation of the CD4 antigen, without depletion of circulating T-cells (7), yet significant immunosuppressive efficacy was provided. Similar observations were made in recipients of the IgG4 humanized molecule (9). Moreover, a clinical trial administering another anti-CD4 mAb, Max.16H5 for treatment of acute rejection has been found to be successful (13), although CD4 antigen modulation without complete depletion was noted (see below). By physically binding to CD4, IgG4 OKTcdr4a may achieve its immunosuppressive effect by blocking CD4 interaction with MHC class II molecules, by inducing a negative CD4 signal to its associated protein kinases, or by blocking a lateral interaction with the T-cell receptor. Whatever the mechanism, these studies emphasized that depletion of CD4+ T-cells is not apparently necessary to accomplish adequate immunosuppression.

In the open-label, non-randomized sequel study to the pilot murine OKT4A trial, 10 CD and 6 LRD primary renal allograft recipients were treated with IgG4 OKTcdr4a. The dosage schedule was either 0.5 or 1.0 mg/kg/d intended to be continued for 12 consecutive days. The first infusion of OKTcdr4a was given within 2 h of transplantation. Recipients were also treated with the standard regimen of CsA, azathioprine, and prednisone. The participating centers were the University of Utah and the Massachusetts General Hospital (manuscript in preparation).

Eleven patients completed the 12 d course. Early discontinuation of OKTcdr4a treatment occurred in 5 CD recipients after 2–7 doses: 3 because of delayed graft function, one because of clinically suspected rejection (not biopsy confirmed), and one because of respiratory failure associated with pancreatitis. This latter patient recovered completely. Since this patient was known to be hyperlipidemic, it was unclear whether the adverse event was related to OKTcdr4a administration.

Two other patient complications not attributed to OKTcdr4a treatment included: a perforated sigmoid diverticulum in one patient at 11 d, and a bacterial pneumonia in one recipient at 45 d following transplantation. Both complications were resolved successfully.

Transient CD4 T-cell depletion was observed post-operatively, but specific CD4 T-cell levels typically were >500 by 1–2 months after transplantation. Peripheral blood CD4 receptor saturation measured by flow cytometry was generally >90% for up to 12 d at both dose levels. Saturation declined to baseline by 1 wk after completion of the 0.5 mg/kg/d treatment. When recipients were given > 5 doses of 1.0 mg/kg/d, CD4 saturation persisted for 7–10 d. Biopsy-proven rejection at 4 and 8 wk after transplantation was observed in 2/11 (18%) patients receiving the full course of OKTcdr4a therapy. Two of 5 patients receiving an incomplete course of OKTcdr4a (both CD) experienced rejection episodes within 10 d of transplantation (1 biopsy proven). All rejection episodes were success-

fully reversed with OKT3 (3) or ATG (1). In summary, the current rejection rate for the patients receiving the full course of OKTcdr4a therapy is 18% (2/11). The length of follow-up is 8 months following transplantation.

OKTcdr4a was well tolerated, and no HAMA was detected. Prior studies have established that most xenogeneic mAbs provoke a recipient response to the mAb constant region, and often to the idiotypic (the variable portion of the immunoglobulin light and heavy chains reactive with the antigenic epitope) region, as well. In our experience, all Cynomolgus recipients of murine mAb developed IgG antimurine antibodies within 15 d of the initial mAb infusion (8). The specificity of these anti-xenogeneic responses was directed to both the anti-idiotypic and anticonstant regions. Similarly, a HAMA response in most human allograft recipients of murine OKT3 has been extensively documented, and it was also frequently observed in the renal allograft recipients of murine OKT4A. Thus, the observation that no HAMA was detected in the OKTcdr4a recipients represents a significant difference from earlier studies.

The consequences of averting the HAMA response are several. It enables allograft recipients to receive effective courses of mAb therapy, for more than the 2 wk period typically employed in current treatment protocols. Averting a HAMA response also provides an opportunity for sequential courses of mAb therapy, with protocols of mAb administration given weekly or monthly. Finally, without a HAMA to the administered mAb, adequate serum levels following either small consecutive doses or a single high dose can be maintained, permitting a prolonged state of therapeutic efficacy. At 10 mg/kg CD4 receptor saturation was 72, 83 and 83% at 14 d after infusion of a single experimental dose to rheumatoid arthritis patients (14). The $t_{1/2}$ of 10 mg/kg was 80 h (14).

This initial clinical experience using OKTcdr4a was encouraging, with no allograft failures observed. It is clear, however, that the dose of 0.5 mg/kg/d was insufficient to accomplish the intended objective: to prevent rejection consistently. Since CD4 saturation was incomplete in the 2 patients who experienced an episode of rejection, other dosage regimens could be required. The Cynomolgus studies have suggested that a single high dose of OKTcdr4a (10 mg/kg) may be the most effective and the most efficient (considering that it is given as a single dose). Aside from the simplicity of the regimen, a single large dose may have the immunological benefit of disarming not only the peripheral blood CD4 T-cells, but also non-circulating cells residing in the lymph nodes and spleen.

### Other anti-CD4 mAb clinical trials

Several other units have also begun to evaluate the clinical potential for mAbs directed against the CD4 determinant. Observations from early trials in renal allograft recipients receiving the murine IgG2a preparations BL4 or MT151 suggested an unusually high incidence of early rejection episodes (15) and no improvement in longer term results. A more recent report summarized the results of a pilot trial using cMT-412 (12). This reagent was constructed by combining the CD4-reactive murine hypervariable regions with the constant region of human IgG1 immunoglobulin. Eleven recipients of heart or heart-lung allografts were treated with standard triple drug therapy plus cMT-412 for the first 3 wk post-operatively. Comparable depletion of CD4 cells to that achieved in ATG-treated patients was observed. In 7 of the recipients, a HAMA response was detected around day 21 post-operatively. After a mean follow-up period of 600 d, ca. 40% fewer rejection episodes and infectious complications were diagnosed in the mAb-treated patients than in the ATG-treated control group. Ten of 11 mAb-treated patients have survived versus 8 of 11 in the control group. The investigators concluded that these observations encourage more extensive studies of this mAb.

Clinical trials using at least 3 other anti-CD4 mAbs are also in progress. One of these, Max.16H5, was initially found to be effective in patients with rheumatoid arthritis (16). More recent evaluation has been undertaken in 10 renal allograft recipients suffering late onset rejection episodes (13). Rejection was diagnosed in these patients 2–7 yr following transplantation. The results of therapy were compared with those achieved in 47 patients treated with ATG, steroids or OKT3 Max.16H5 mAb induced significant modulation of the CD4 antigen which reverted to pre-treatment levels within 2–10 d after cessation of therapy. Depletion of CD4+ cells was partial and observed in only 8 of 10 patients. A detectable HAMA response was not observed in any of the recipients. At 1 yr following Max.16H5 treatment, 7 of 10 allografts had good to excellent function which was comparable to the results in conventionally treated patients. However, the investigators reported fewer early side-effects and fewer subsequent viral infections in the anti-CD4 treated patients prompting them to conclude that this may be an especially attractive and novel therapeutic strategy for late onset rejection therapy.

Another anti-CD4 mAb, BF5, initially studied in patients with rheumatoid arthritis has been administered together with an anti-CD25 mAb and conventional suppression to 10 renal allograft recipients

(17). The investigators observed only mild rejection episodes in 3 patients, but 3 allografts were lost from arterial or venous thromboses. Since the relationship of this complication to the mAb therapy was unclear, the authors proposed further studies in a randomized trial.

Finally, another humanized anti-CD4 mAb, prepared by Centocor Corp. (G. Fathman, personal communication), has also entered clinical trials. This agent, like OKT3, does not cross-react with lower primates, and therefore no pre-clinical observations in allograft recipients are available. In Phase I studies, marked and prolonged CD4+ T-cell depletion has been noted, leading some to suggest that an AIDS-like syndrome could possibly result from such therapy. To date, no evidence of such toxicity has been reported. It remains too early for definitive evaluation of the efficacy or toxicity of this mAb.

## Conclusions

These numerous clinical trials, and undoubtedly others which are as yet unreported, emphasize the intense current interest in anti-CD4 mAb immunosuppression. Enthusiasm for this approach to rejection management has been fuelled by the pre-clinical studies indicating that donor specific tolerance with only minimal concomitant compromise of the host's general immune system can be induced with anti-CD4 immunomodulation. It appears that temporary disabling or depletion of CD4+ T-cells during allo-antigen presentation provides the appropriate conditions for the foreign specificities to be perceived as self-antigens. The mechanism involved with the subsequent long-term donor specific hyporeactivity has been variably attributed to clonal anergy that results from antigenic engagement in the absence of a sufficient "second signal" (18), clonal deletion (19), or antigen-specific lymphocyte suppression (20). Whatever the true mechanism proves to be, the impressive results observed in many rodent models and some non-human primate studies suggest that CD4 mAbs will probably play a prominent role in future immunosuppressive protocols. Current reports emphasize, however, that the clinical experience to date remains too preliminary to determine whether the promise of such an approach will be fulfilled in humans.

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# Manipulation of xenogeneic skin and/or renal graft survival in the rat–mouse concordant combination by portal vein pretransplant transfusion

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**Abstract:** We have examined whether portal venous pretransplant transfusion, which has been shown to produce prolongation of rodent vascularized (small intestine, kidney) and nonvascularized (skin) allografts, in the absence of other nonspecific immunosuppression, can produce similar graft prolongation in animals receiving vascularized or nonvascularized xeno- (not allo-) grafts.

Rat kidney or skin grafts were transplanted into mice after portal venous pretreatment with rat or mouse spleen cells. Animals in some groups received additional immunosuppressive regimens including drug therapy (methotrexate, cyclosporin A) or monoclonal antibody treatment (anti-CD4, anti-CD8). Animal survival and serum creatinine was followed daily, and lymphoproliferation, cytokine production (including cytokine mRNA in grafted mice) and anti-xenograft antibody production was measured at distinct time points postgrafting. Both portal venous pretransplant transfusion and anti-CD4 monoclonal antibody treatment led to increased graft survival. However, unlike the rodent allograft model, graft survival in these animals was not simply explained by altered Th1/Th2 ratios. Other mechanism(s), possibly including xenoantibody production, are likely of importance in the regulation of xenograft rejection.

## Introduction

As transplantation becomes a more general option for a variety of acute and/or chronic medical problems, a major limitation has been realized in organ availability for the number of potential recipients. Accordingly, transplantation across species, xenotransplantation, has been considered a fruitful avenue for further exploration. Early complement activation (on graft endothelium), probably associated with so-called naturally occurring xenoantibodies, leads to hyperacute rejection and relatively immediate (minutes to hours) graft failure in such models, particularly for grafts between widely disparate (discordant) species. Inactivation of complement, and/or depletion of natural antibodies in the recipient, is associated with diminished or absent hyperacute rejection.[1] Antibodies directed to xenoantigens also play an important role in later events in acute/chronic graft destruction.[1–5] Complement activation is probably less important in concordant xenograft rejection, but details of the immune mechanisms involved in rejection in these models remain obscure.

Using a variety of other manipulations, including transgenic mice, antibody and/or drug-treated mice and mice bearing different mutations (*bg/bg*, *nu/nu*, SCID, etc.), attempts have been made to explore other mechanisms involved in acute and/or chronic rejection in xenograft recipients of concordant and discordant grafts.[6–12] There are some data suggesting that Th2-type cells may be preferentially activated by xenoantigens, a finding in keeping with the notion of a role for antibody responses in xenorejection.

Our laboratory has previously reported on the role of different pretransplant transfusion protocols in producing

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prolongation of allogeneic skin grafts in mice, or allogeneic renal and small bowel transplants in rats. In association with delayed graft rejection in these models there was a consistent alteration in the cytokine production profiles of activated T cells obtained from treated animals. In particular, portal venous immunization was found to be remarkably effective as a means of adjusting the balance of Th1/Th2 activation towards Th2-type cells, as well as prolonging graft survival. There are studies suggesting a preferential activation of Th2-type cells in concordant xenografts,[13,14] and independent experiments which infer a role for blocking accessory molecule signalling (a technique we used to facilitate preferential activation of Th2-type cells[15]) in enhancing concordant heart xenograft survival.[12]

The experiments described in this report have explored whether portal venous pretransplant transfusion can modify rejection of concordant rat skin or renal xenografts in mice, and whether alterations in Th activation and xenoantibody production are associated with this treatment. Altered antibody-mediated cytoxicity towards rat xenoantigens in mice receiving rat skin or renal grafts has been investigated with a focus on those reactivities detected using rat endothelial or rat lymphocyte blast targets.[16,17]

## Materials and methods

### Animals

C3H/HEJ mice were obtained from Charles River (Canada); Lewis (Le) or Brown Norway (BN) rats were from Sprague Dawley (Indianapolis, USA). Male animals, 8–10 weeks of age, were used throughout. Animals were kept five per cage (mice) or two per cage (rats) and allowed food and water ad libitum.

### Monoclonal antibodies

The following monoclonal antibodies (mAbs) were used: anti-CD4 (GK 1.5, ATCC); anti-CD8 (2.43, ATCC); anti-IL-2 (S4B6, ATCC); anti-IL-4 (11B11, ATCC); anti-IFNγ (XMG1.2, ATCC); anti-IL-10 (SXC-2, ATCC). All mAbs were grown as ascites in BALB/C *nu/nu* mice and purified by ammonium sulphate precipitation and resuspension in phosphate-buffered saline (PBS) before use. Anti-CD4/-CD8 were injected intravenously into mice (250 μg per mouse) 4, 2 and 0 days before use. In control studies this treatment schedule produced ≥95% depletion of $CD4^+$ or $CD8^+$ cells (assayed by FACS analysis) compared with untreated mice. *In vitro* lymphokine assays used 5 μg/ml of the Ig (antilymphokine) preparations. These concentrations of mAbs tested in the lymphokine assays described *in vitro* (see below) produced relatively equivalent amounts of inhibition (≥10 units of lymphokine neutralized).

### Portal vein immunization

Irradiated (2000 R) rat (or mouse as control) spleen cells ($100 \times 10^6$) were injected into anaesthetized animals as described elsewhere.[18]

### Xenogeneic skin or renal grafts

Rat tail skin grafts (1 $cm^2$) were applied to mice under anaesthesia as described earlier.[19] Protective coverings were removed from the grafts at 7 days and the grafts inspected daily for signs of rejection. A minimum of six mice were used per group as skin graft recipients.

Kidneys from Le donors were transplanted to C3H/HEJ recipients following standard techniques,[20] with venous drainage to the inferior vena cava. The ipsilateral host kidney was removed at transplantation, and the remaining host kidney was removed on the second postoperative day. All animals were fed a regular laboratory diet beginning on the first postoperative day, and daily weights recorded. For the renal transplant studies blood samples were obtained from the tail at 36-hour intervals to follow serum creatinine using a kit purchased from Sigma Chemicals (St Louis, MO). All experimental groups contained six mice.

Some groups also received additional treatment as follows: cobra venom factor (CVF; Sigma), 5 μg per mouse (i.p.), daily from time of transplantation; cyclosporin A (CsA; Sandoz, Canada), 0.1 mg per mouse (i.m.), alternate days ×4 from time of transplantation; methotrexate (MTx), 5 μg per mouse (i.m.) daily from time of transplantation; heat inactivated, pooled (≥20 adult Le rats) rat serum, 0.75 ml per mouse (i.v.) daily from time of transplantation.

The rationale for these treatments was to explore any potential role for complement activation and naturally occurring xenoantibodies in this concordant xenograft model (use of CVF and rat serum), to examine the role of 'conventional' T cell immunosuppressive regimes (CsA) directed preferentially at IL-2 producing T cells,[21] and to investigate whether broader-spectrum antiproliferative drugs (anti-B/-T cell, MTx) would improve xenograft survival.

### Preparation of cells and lymphokine assays

Spleen cell suspensions were prepared aseptically from individual animals. For mixed lymphocyte cultures responder cells were stimulated with irradiated (2000 R) spleen stimulator cells in triplicate in minimal essential medium (alpha modification) supplemented with 2-mercaptoethanol and 10% fetal calf serum (αF10). Supernatants were pooled at 72 hours from replicate wells and assayed in triplicate for lymphokine production as described below. In some experiments the culture wells then received 1 μCi per well of $^3$H-TdR (thymidine) and proliferation was measured by harvesting the contents of the well 14 hours later and counting in a well-type beta-counter.

IL-2, IL-4, IL-10 and IFNγ cytokine assays were performed as described in previous reports.[22]

### Preparation of RNA

Different sources of lymphoid tissue from skin-grafted mice were homogenized in a glass homogenizer in 8 M guanidine hydrochloride containing 0.1 M EDTA and 1/10 volume of 3 M sodium acetate.[23] Tissue was pooled from three mice per group used. Subsequent RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described in an earlier publication.[24]

### Antibody-mediated cytotoxicity assays

Both rat lymphocyte concanavalin A (Con A) blasts and endothelial cells (EC) were used as targets. EC monolayers were prepared as described elsewhere.[25] In brief, fetal hearts were pooled from three 24-hour old Le rats, minced on wire mesh screens in PBS, and the released cells discarded. Stromal tissue of each source was enzymatically digested in

0.5% type 1 collagenase and 1% dispase (Sigma) for 45 minutes at 37 °C. Cells were filtered through nylon mesh (100 μm diameter), washed twice in αF10, and centrifuged over discontinuous Percoll gradients. Cells localized to the interface between densities 1.044 and 1.046 g/ml were collected. The isolated cells were resuspended in αF10 and $2 \times 10^6$ cells added in 4 ml of αF10 to 1% gelatin-coated culture dishes (60 mm; Costar, Cambridge, MA) before incubation for 4 hours at 37 °C. Nonadherent cells were washed off with prewarmed medium and fresh medium supplemented with 100 μg/ml bovine endothelial mitogen (Biomedical Technologies Inc., Stoughton, MA) and 10 U/ml heparin was added. Primary cultures were fed at 4–5-day intervals and, upon confluence, cells were recovered by digestion with PBS containing 10 mm EDTA and 0.5% collagenase. EC used in the assays described were in their first passage *in vitro*.

Rat peripheral lymph node lymphocytes were pooled from three normal Le donors and stimulated for 48 hours in culture with 5 μg/ml Con A. Both EC and Con A blast cells were washed three times with warm αF10 and recultured for 8 hours in 2.5 ml of αF10 ($2.5 \times 10^6$ cells) with 5 μCi of $^3$HTdR-proline (New England Nuclear). Each cell preparation was again washed three times with αF10 and used in cytotoxicity assays as follows.

Heat-inactivated (56 °C for 30 minutes) serum pooled from different groups of transplanted mice (minimum of six mice per group) was tested in triplicate at varying concentrations (from 1% to 20%) with $2.5 \times 10^4$ $^3$H-TdR-labelled target cells and either 10% baby rabbit complement (Cedarlane Labs, Hornby, Ontario) for a complement-mediated cytotoxicity assay (CDC), or with a 150-fold excess of murine spleen cells (ADCC assay) in a final culture volume of 300 μl. Cultures were incubated at 37 °C for 90 minutes (complement lysis) or 4 hours (ADCC), and an aliquot (100 μl) of the supernatant was removed for counting in a gamma-counter. Control cultures (spontaneous $^3$H-TdR release) contained medium only. Further cultures were incubated with detergent to measure maximum releasable $^3$H-TdR. Data are expressed as percentage specific cytotoxicity: [(experimental − control)/(maximum released − control)] × 100.

## Results

### Portal venous immunization and cytotoxic drug treatment prolongs rat skin xenografts in mice

In preliminary studies we found that CVF and rat serum were essential to produce reproducible survival in concordant renal xenotransplant recipients beyond 96 hours (unpublished), and that additional treatment with nonspecific cytotoxic drugs (CsA and MTx) led to some further significant prolongation in graft survival. Accordingly, experiments designed to investigate the effect of other more immunologically specific treatments included these treatments also to the experimental animals. To assess the role of pretransplant transfusion on xenogeneic skin graft rejection, C3H/HEJ mice were injected intravenously or portal venously with Le splenocytes (pooled from four donors), with or without cytotoxic drugs. Data pooled from three experiments of this type are shown in Figure 1 (values in parentheses indicate the total number of animals used in these groups).

Either portal venous injection alone, or combination cytotoxic drug treatment alone, afforded significant prolongation of xenograft survival in this model. However, optimum survival was achieved using both portal venous immunization and cytotoxic drug treatment. Pretreatment of animals by intravenous immunization led to more rapid graft rejection (see also ref. 26).

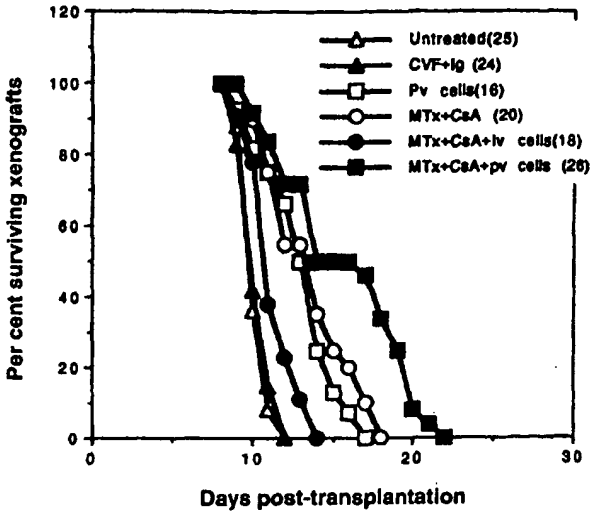


**Figure 1** Skin graft rejection of Lewis rat skin in groups of C3H/HEJ mice treated before/after transplantation as shown (see text for more details). □, ○: $p < 0.05$; ■: $p < 0.02$; Mann–Whitney *U*-test.

In a separate study we examined the antigen specificity of skin graft prolongation afforded by drug and/or Le spleen cell (portal venous) pretreatment. Groups of 12 mice were treated as above, and six mice per group were grafted with either Le or BN skin grafts. Graft survival for these groups is shown in Figure 2. Cytotoxic drug treatment alone prolongs survival of both Le and BN grafts, whereas portal venous immunization, alone or in combination with cytotoxic drugs, imparts significant antigen specificity to the graft prolongation such that Le, but not BN, grafts show enhanced survival compared with the group treated with cytotoxic drugs alone. In reciprocal studies (not shown) preimmunization with BN cells led to increased survival of BN compared with Le grafts.

### Role of CD4$^+$/CD8$^+$ cells in xenograft rejection

In order to investigate the role of different T cell subsets in rejection of rat skin xenografts, groups of six mice were treated for 4 days before grafting with anti-CD4/-CD8 mAbs. In addition, some groups received portal venous pretreatment with irradiated Le spleen cells. All animals except an untreated control group received CVF and Ig. Data pooled from three such studies are shown in Figure 3 (numbers in parentheses represent total number of animals per group).

Portal venous pretreatment alone caused significant graft prolongation. Anti-CD4 also produced enhanced graft survival, although anti-CD8 treatment produced no significant increase survival beyond the control group. Portal venous treatment along with anti-CD4 produced a synergism in graft prolongation, while combination anti-CD8 and portal venous treatment a little further enhanced graft survival beyond that seen with portal venous treatment alone; no additional effect

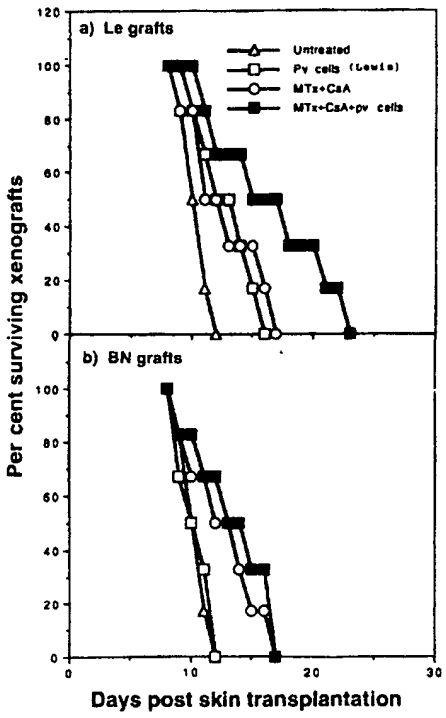


**Figure 2** Skin graft survival of Lewis (panel a) or Brown Norway (panel b) skin grafts in groups of six C3H/HEJ mice treated as shown (see also Figure 1). Mice receiving portal venous pretransplant transfusion (pv cells) received $100 \times 10^6$ irradiated Lewis spleen cells 36 hours before grafting. ■: $p < 0.05$; Mann–Whitney *U*-test, panel a.

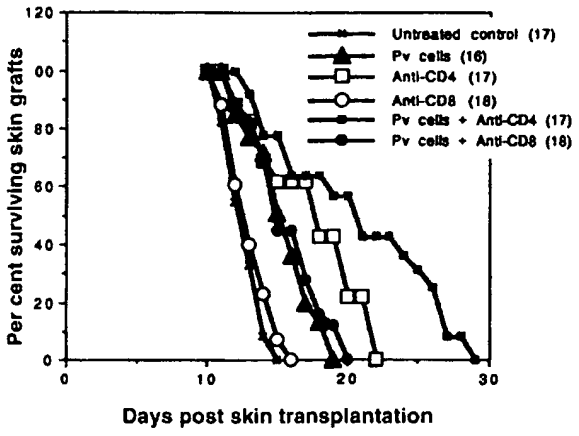


**Figure 3** Affect of treatment with anti-CD4/-CD8 on rat skin graft survival in groups of C3H/HEJ mice (see also text and legend to Figure 1). □, ●, ▲: $p < 0.05$; ■: $p < 0.02$; Mann–Whitney *U*-test.

was seen with intravenous cells and anti-CD4/-CD8 (unpublished, but see Table 1).

## Cytokine synthesis from restimulated cells of xenografted mice

We described earlier a diminished IL-2/IFNγ production from portal venously immunized animals, with persistence of IL-4/IL-10 production, which correlated with a specifically decreased ability to reject skin allografts.[27] We suggested that this phenomenon resulted from a relative decrease in Th1 (versus Th2) activation. To investigate the operation of a similar mechanism in the xenograft model the following study was performed.

Groups of six mice received Le skin grafts along with other treatments as shown in Figure 1 and/or Figure 3. At 7 days post-transplantation three animals were pooled for each group and spleen cells recultured in microtitre wells with irradiated Le stimulator cells. Supernatants were tested in lymphokine assays, and cells for proliferation, as described in 'Materials and methods'. Data for one of three studies are shown in Table 1.

Portal venous pretreatment along with either anti-CD8 mAb, or MTx + CsA, leads to a significant change in cytokine profiles on restimulation of lymphoid cells taken from the grafted animals, with production of predominantly Th2-type cytokines (IL-4/IL-10) rather than the Th1 cytokines (IL-2/IFNγ) seen in other groups. These same groups show significant prolongation of survival of skin grafts compared with controls (see Figures 1 and 3). Note that while anti-CD4 treated mice (with/without portal venous treatment) show prolonged graft survival (Figure 3), cells from these animals produced minimal detectable Th1 or Th2 cytokines after restimulation (Table 1). Presumably graft survival in anti-CD4-treated mice is not necessarily dependent on a 'switch' to Th2 activation. For portal venous treatment to lead to altered cytokine profiles in restimulated splenocytes, mice must contain intact $CD4^+$ cells (penultimate row, Table 1; see also ref. 27).

## Cytokine mRNA in treated mice receiving rat skin xenografts

We examined the expression of mRNAs for IL-2, IL-4, IL-10 and IFNγ in mice receiving rat skin grafts after treatment with immunosuppressive drugs, portal venous preimmunization, or mAbs to murine $CD^{4+}$ or $CD8^+$ cells (see Table 1). Draining lymph nodes were removed from animals 120 hours after grafting for this analysis. PCR data from one such study (of four) are shown in Figure 4.

Control mice showed IL-2 and IFNγ mRNAs in the draining nodes. Only portal venously treated mice, with/without additional treatment with MTx + CsA or anti-CD8 antibody, showed significant levels of IL-4 and IL-10 mRNA. Minimal cytokine mRNAs were detected in anti-CD4-treated mice.

## Rat renal xenograft survival and altered cytokine synthesis after portal venous pretransplant transfusion

We next examined a vascularized xenograft model in which mice received rat renal grafts after portal venous transfusion with/without MTx + CsA. All mice except a control group received daily injections of CVF and rat serum. Cytokines

**Table 1** Pretransplant portal venous infusion enhances IL-4 production in xenografted mice

| Treatment[a] | $^3$H-TdR[b] | Cytokine synthesis[c] | | | |
|---|---|---|---|---|---|
| | | IL-2 | IL-4 | IFNγ | IL-10 |
| None | 9875 ± 2340 | 7.3 ± 1.1 | 2.2 ± 0.7 | 95 ± 10 | ≤1.0 |
| CVF + Ig only | 8760 ± 1875 | 7.0 ± 1.8 | 2.0 ± 0.6 | 90 ± 15 | ≤1.0 |
| IV cells | 8895 ± 2245 | 7.5 ± 1.6 | 2.1 ± 0.5 | 95 ± 15 | ≤1.0 |
| PV cells | 4355 ± 780 | 3.8 ± 1.1 | 6.1 ± 0.9* | 65 ± 10 | 4.5 ± 1.5* |
| MTx + CsA | 5435 ± 1255 | 2.0 ± 0.9 | 1.6 ± 0.8 | 55 ± 10 | ≤1.0 |
| MTx + CsA + IV cells | 5055 ± 1340 | 2.2 ± 0.7 | 1.7 ± 0.7 | 60 ± 10 | ≤1.0 |
| MTx + CsA + PV cells | 2450 ± 695 | 1.9 ± 0.5 | 6.6 ± 1.4* | 35 ± 10 | 7.5 ± 2.0* |
| Anti-CD4 | 2345 ± 810 | 1.5 ± 0.4 | 0.9 ± 0.3 | 30 ± 10 | ≤1.0 |
| Anti-CD8 | 8895 ± 1670 | 7.0 ± 1.6 | 1.8 ± 0.5 | 85 ± 15 | ≤1.0 |
| Anti-CD4 + IV cells | 1655 ± 650 | 1.2 ± 0.4 | 0.7 ± 0.3 | 30 ± 10 | ≤1.0 |
| Anti-CD4 + PV cells | 1565 ± 450 | 1.0 ± 0.4 | 0.8 ± 0.4 | 25 ± 10 | ≤1.0 |
| Anti-CD8 + IV cells | 8090 ± 1550 | 8.0 ± 1.7 | 2.0 ± 0.6 | 90 ± 15 | ≤1.0 |
| Anti-CD8 + PV cells | 2325 ± 670 | 1.7 ± 0.5 | 6.0 ± 1.6* | 30 ± 10 | 5.5 + 1.0* |

[a] Groups of six C3H/HEJ mice per group received Le tail skin grafts (1 cm$^2$). A control group (first row) received no other treatment. All other animals received CVF and Ig (rat serum) as described in the 'Materials and methods'. CsA (0.1 mg per mouse i.m.) on alternate days for 4 doses beginning on the day of transplantation, and MTx (5 μg per mouse i.m. daily from the day of transplantation) were injected in some groups. PV or IV cells: $100 \times 10^6$ irradiated (2000 R) Le rat spleen cells via portal vein or lateral tail vein (36 hours before transplantation). Some groups received intravenous anti-CD4 or anti-CD8 (250 μg per mouse) on days −4, −2 and on the day of transplantation. Spleen cells were pooled from three mice per group at 7 days and restimulated in triplicate in microtitre plates with irradiated Le spleen cells.

[b] Arithmetic mean (±SD) of $^3$H-TdR incorporation into cultures at 72 hours. c.p.m. in nonstimulated spleen cultures averaged over all groups was 1245 ± 435.

[c] Cytokine synthesis *in vitro* at 72 hours from cultures described in footnote 'a'. Data represent U/ml (arithmetic mean ±SD) from triplicate determinations using CTLL-2 or CT4.S, except for IFNγ where data are expressed as ng/ml (ELISA assay using rIFNγ as standard). No detectable cytokines were detected in supernatants of nonstimulated cultures (data not shown).

* $p \leq 0.05$ (ANOVA and protected *t*-test compared with untreated control).

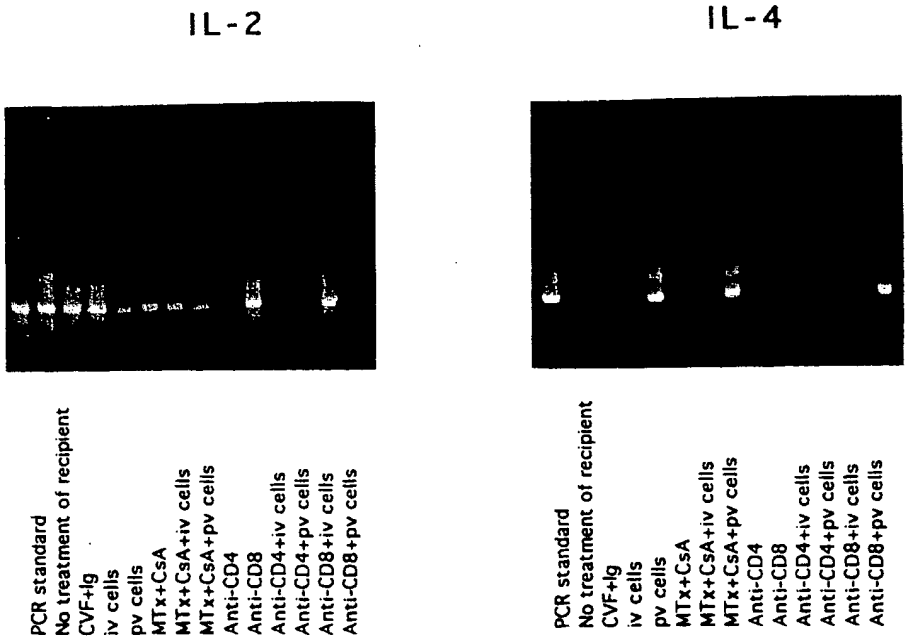


**Figure 4** PCR cytokine products in draining lymph nodes of C3H/HEJ mice at 120 hours post Le rat skin grafting, using groups of animals treated as shown in rows 1–13 of Table 1. The first lane in each panel represents a positive control for the relevant PCR product.

were measured from 72-hour cultures of spleen cells taken from six mice per group at 5 days post-transplantation. Survival data pooled from a minimum of 15 mice per group over 5 months are shown in Table 2.

In support of the studies with skin xenografts, optimal animal (renal graft) survival was seen with mice receiving portal venous transfusion and immunosuppressive drug treatment (along with CVF and Ig). Once again (see Table 1), all animals receiving portal venous transfusion showed diminished IL-2 and IFNγ production and enhanced IL-4 and IL-10 synthesis after restimulation *in vitro*, which was correlated with improved animal (graft) survival in these groups.

### Complement- or cell-mediated antibody-dependent cytotoxicity in mice with rat skin xenografts

Groups of six per group C3H/HEJ mice received Le rat tail skin grafts (or renal grafts) along with the treatments outlined in Table 1. Samples of blood (300 μl) were obtained from the tail of individual mice in each group under anaesthesia at 7 (renal grafts) or 13 and 20 (skin grafts) days post-transplantation, and serum was collected and stored at −70 °C until use. Sera were tested in duplicate in a final volume of 250 μl with either absorbed baby rabbit complement (CDC) or pooled normal mouse spleen cells (ADCC). Data in Figures 5 and 6 show the group mean percentage CDC and ADCC respectively using a 1:40 dilution of each serum taken from skin-grafted mice; data in Figure 7 show equivalent data from the mice with rat renal grafts.

Note that serum was tested in these different models at different times post-transplantation, because of the restrictions imposed by animal (graft) survival in the two models. Nevertheless, several features can be noted from these figures. Both qualitatively and quantitatively, cytotoxicity for EC is independent of cytotoxicity for lymphoid targets, particularly for vascularized grafts. Thus CDC or ADCC for EC is reduced approximately 50% in the MTx + CsA + portal venous transfusion group compared with untreated grafted controls, while cytotoxicity for lymphoid cells is marginally increased in this same group. Generally greater cytotoxicity was seen with ADCC than with CDC, regardless of the model system. The apparently greater cytotoxicity seen with skin graft recipients compared with renal graft recipients (Figures 5 and 6 versus 7) may be a result of the different times at which serum was harvested post-transplantation. When serum was obtained from skin graft recipients at 7 days, quantitatively similar levels of cytoxicity (to lymphoid cells) were seen as with the renal graft recipients (data not shown).

Finally, comparison with graft (animal) survival data (see Figures 1–3) showed no obvious correlation between ADCC or CDC and survival in these various groups, with the exception perhaps for the decreased ADCC to EC seen in renal xenograft recipients treated with combined MTx + CsA + portal venous transfusion (see Figure 7, panel b). In a separate study in a model in which skin graft survival was prolonged by anti-CD4 antibody (see Table 1), there was again minimal perturbation of ADCC (or CDC) for EC/lymphoid blasts at days 13/20 post-transplantation, despite the marked changes in graft rejection in these mice (ADCC at 13 days for EC/lymphoid blasts in untreated/anti-CD4 treated: 15 ± 3.5, 63 ± 8.4 and 13 ± 2.7, 45 ± 6.3, respectively).

## Discussion

There is some belief that the next major revolution in transplantation will come with the advent of successful routine xenografting. The reconstituted (with xeno peripheral blood lymphocytes) SCID mouse has been a popular model for exploration of discordant xenograft transplantation.[8,9] More recently there have been studies focusing on therapy designed to compare and contrast allo- and xenorejection, as well as to manipulate cell-mediated (acute/chronic) graft rejection and hyperacute rejection in more conventional models.[13,14]

Many of the studies reported to date have focused on discordant xenograft models where complement-mediated hyperacute rejection is a major problem.[2,17,28–30] Decreasing antibody-mediated complement activation prolongs discordant xenograft survival.[1,3–5,28,31–33] Antibodies to antigens expressed, or overexpressed after 'upregulation', on graft EC are of relevance to the rejection process.[17,29,32,34,35] Given these problems of hyperacute rejection, fewer studies in discordant xenograft models have explored the phenomena involved in later stages (acute/chronic) of rejection. Intravenous injection

**Table 2** Graft survival and cytokine synthesis after xenogeneic renal transplantation

| Group treatment[a] | Mean animal survival time (days) | Cytokine synthesis (U/ml)[b] | | | |
|---|---|---|---|---|---|
| | | IL-2 | IL-4 | IFNγ | IL-10 |
| No transplant | – | 5.2 ± 1.3 | 1.0 ± 0.3 | 60 ± 10 | ≤1.0 |
| None | 4.8 ± 1.5 | 8.4 ± 1.7 | 1.2 ± 0.3 | 95 ± 15 | ≤1.0 |
| MTx + CsA | 5.0 ± 1.6 | 2.7 ± 0.7 | 1.0 ± 0.3 | 40 ± 10 | ≤1.0 |
| CVF + Ig | 7.9 ± 1.1 | 7.2 ± 2.0 | 0.9 ± 0.2 | 95 ± 15 | ≤1.0 |
| CVF + Ig + IV cells | 6.5 ± 1.2 | 7.9 ± 1.9 | 1.0 ± 0.3 | 85 ± 15 | ≤1.0 |
| CVF + Ig + PV cells | 9.8 ± 1.7 | 2.5 ± 0.8 | 2.5 ± 0.8* | 45 ± 10 | 3.0 ± 1.0* |
| CVF + Ig + MTx + CsA | 9.8 ± 1.6 | 3.0 ± 0.8 | 0.9 ± 0.4 | 35 ± 10 | ≤1.0 |
| CVF + Ig + MTx + CsA + IV cells | 9.0 ± 1.5 | 3.1 ± 1.0 | 1.0 ± 0.3 | 35 ± 10 | ≤1.0 |
| CVF + Ig + MTx + CsA + PV cells | 14.0 ± 2.1* | 2.1 ± 0.8 | 3.6 ± 0.8* | 25 ± 5 | 5.0 ± 1.0* |

[a] C3H/HEJ mice received rat renal grafts as described in 'Materials and methods', along with pretransplant transfusion via the portal vein or lateral tail vein of 100 × $10^6$ irradiated Le spleen and other treatment as shown. Data are pooled from a minimum of 18 mice per group.

[b] IL-2, IL-4, IFNγ and IL-10 were assayed in triplicate as described in Table 1. Data represent arithmetic means (±SD) for six mice per group sacrificed at 5 days post-transplantation.

* $p < 0.05$ compared with untreated (or nontransplanted) controls in rows 1 or 2.

of transgenic (HLA-B27) cells into mice immunizes lymphocytes against B27, while the same procedure induced tolerance in the allogeneic (transgenic) combination.[36] $\beta_2$-Microglobulin-deficient (knockout) mice ($CD8^-$) still rejected pancreatic xeno- but not allografts,[11] implying a crucial role for $CD4^+$ cells in xenorejection.

Our studies have focused on a concordant xenograft model (the equivalent in human of nonhuman primate to human grafts), and invite comparison with earlier data in an allograft model using portal venous transfusion.[22,27,37–41] There are reports that hepatocytes suppress delayed-type hypersensitivity responses in xenografted animals,[10] that infusion of rat islet grafts portal venously into mice with antilymphocyte serum leads to antigen-specific increased (90-day) survival of the WF graft with rejection of a Lewis graft,[42] and that comparison of cytokine production (mRNA) after *in vitro* xeno/allo-mixed lymphocyte reaction suggests a preferential activation of Th2 cells in xeno combinations.[13,14] More recent observations in proislet discordant xenografts (pig-to-mouse) also support a role for Th2-type cytokines in prolonging xenograft survival.[43]

There is evidence that T cells responsible for xenorejection 'follow some of the same rules' for activation as those involved in allorejection. Thus a role for costimulatory molecules in the regulation of T cell activation for xenoantigen recognition was inferred from evidence that CTLA4-Ig blocked discordant xenoreactions (mouse antihuman islet rejection) *in vivo*,[44] and that CD4/CD8 'blocking' antibodies could assist development of tolerance in concordant heart-xenografted mice.[12] We reported that neither LFA-1/ICAM-1 nor CTLA4-Ig/B7 was critical for delivery of 'anergizing' signals in portal venously treated animals.[24]

In the data reported in this study we initially asked whether rat skin (or renal) xenograft survival was prolonged in mice treated to avoid early complement activation (CVF and rat Ig infusion), as well as receiving nonspecific (MTx + CsA, or anti-CD4/-CD8 antibodies) or specific (portal venous pretransplant transfusion) immunosuppression. Perhaps somewhat surprisingly for this concordant graft model, it seems that CVF and rat Ig combined did in fact produce a significant, albeit modest, increase in survival of vascularized grafts (Table 2). We do not yet know if the rat serum given to these animals binds to preformed antibody, or to antibodies produced shortly after transplantation.

Combination therapy using portal venous transfusion with MTx + CsA or anti-CD4 provided optimal antigen-specific graft prolongation in our model, although nonspecific increased survival was also seen using MTx + CsA or anti-CD4 only. Animals receiving portal venous pretreatment, alone or in combination with MTx + CsA or anti-CD8, showed specifically enhanced activation of Th2-type cells as measured by *in vitro* IL-4 and IL-10 production on restimula-

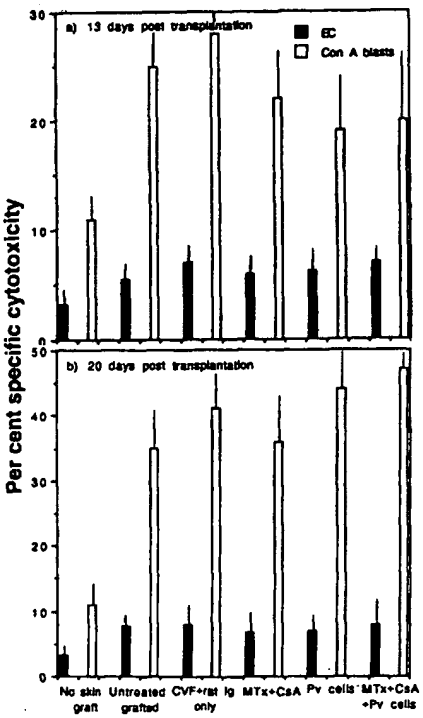


**Figure 5** Arithmetic mean (±SD) of complement-mediated cytotoxicity (CDC) using serum from different groups of treated C3H/HEJ mice (see abscissa) at 13 days (upper panel) or 20 days (lower panel) post-transplantation of Le rat tail skin. A minimum of 12 sera per group were tested individually and data pooled within each group. Cobra Venom Factor (CVF), heat-inactivated rat serum (rat Ig) and methotrexate (MTx, 5 μg per mouse) were injected daily from the day of transplantation. Cyclosporin A (CsA, 0.2 mg per mouse) was injected every 48 hours. $100 \times 10^6$ irradiated rat spleen cells were injected via the portal vein 36 hours before grafting (pv cells). Target cells ($2.5 \times 10^4$ per assay well) were endothelial cells (EC) from neonatal rat hearts or 48-hour Con A rat spleen cell blasts.

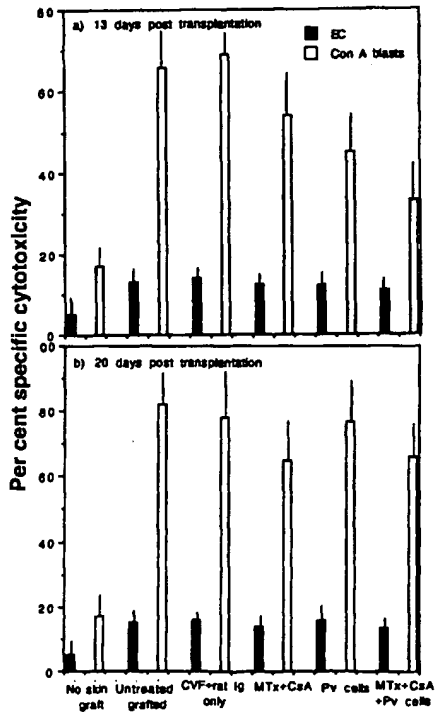


**Figure 6** Cell-mediated antibody dependent cytotoxicity (ADCC), with effector/target of 150:1, using pooled mouse spleen effector cells (minimum of three donors per experiment) and targets as in Figure 5. Sera were the same as in Figure 5.

tion, or by cytokine mRNAs assessed *in vivo* (Table 1 and Figure 4). These effects were restricted to animals receiving antigen challenge via the portal vein, a phenomenon described elsewhere also for skin allografts: no such prolongation of graft survival, or alteration in cytokine production profiles, occurred in animals receiving antigen challenge via the lateral tail vein (intravenous route). Nonspecific inhibition of all cytokine synthesis was associated with the increased graft survival following MTx + CsA alone, or after anti-CD4 treatment. Nevertheless, portal venous treatment combined with anti-CD4 antibody afforded the longest skin xenograft survival (see Figure 3). We would conclude, therefore, that the mechanism(s) responsible for graft prolongation in mice receiving anti-CD4 and portal venous pretreatment is quite different from that associated with portal venous treatment alone or combined with MTx + CsA, where increased Th2 cytokine synthesis may be important, as was reported for allografted mice.[15] Whether activation of a $\gamma\delta TcR^+$ ($CD4^-$) suppressor cell population (see ref. 22), acting via a mechanism independent of the cytokines measured, may help explain the prolonged survival in anti-CD4 treated, portal venously immunized, mice is currently under investigation.

Even in the renal concordant xenograft model, where in discordant xenografts hyperacute rejection can be modified by CVF and Ig,[2,31,33] prolongation of survival with MTx + CsA, along with portal venous transfusion, was correlated with decreased IL-2 and IFNγ production and enhanced IL-4 and IL-10 production (Table 2). Delivery of antigen via the intravenous route did not produce graft prolongation or alter cytokine production profiles.

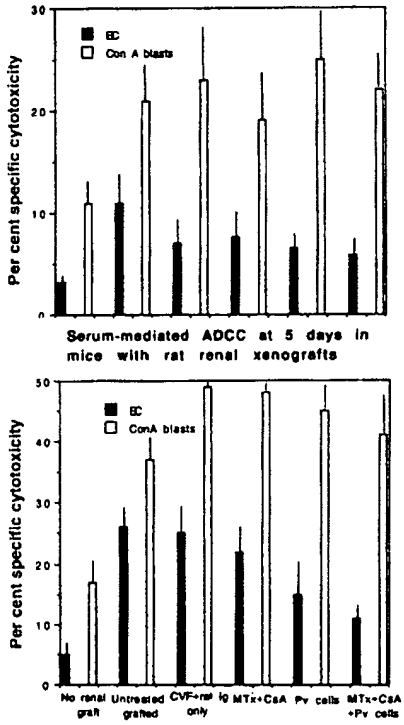


**Figure 7** CDC (panel a) or ADCC (panel b) using serum obtained at 5 days post-transplantation of groups of C3H/HEJ mice with adult Lc renal grafts. A minimum of eight sera per group were tested individually and data pooled within groups. See text and legend to Figures 5 and 6 for more details.

The contribution of antibody responses to the rejection of discordant or concordant xenografts has been investigated by a number of groups.[4,6,45] In our analysis, with the exception of mice receiving vascularized renal xenografts after portal venous treatment and MTx + CsA, where decreased antibodies to EC (but not to lymphoid blasts) correlated with improved survival, no evidence for a major unique role for antirat antibodies in graft or tissue rejection could be seen (Figure 7). In keeping with other studies,[16,17] there was no correlation between cytotoxicity to EC and a lymphoid blast target in the sera tested (particularly for mice receiving vascularized grafts); nor was there any consistent correlation between cytotoxicity measured by CDC versus ADCC, although, in general, lysis in ADCC assays was quantitatively superior to that measured by CDC. In all groups antibody levels subsided after graft rejection, although again there was no consistent correlation between declining cytotoxicity to EC or lymphoid blasts. Perhaps most intriguing of all, we noted at least transient graft survival in both renal and skin xenogeneic models at times when demonstrable xenoantibodies to EC were present. Whether some blood vessels in these grafts were of host, rather than donor, origin was not determined.

A complete understanding of graft rejection in these models is likely to be dependent upon a more clear-cut assessment of the interaction of both cell-mediated and antibody-mediated phenomena (see also ref. 6). However, as defined by these studies, multiple mechanisms probably exist for prolongation of concordant xenograft survival. Our data support a role for pretransplant immunization via the portal vein as one route by which xenograft survival may be prolonged, with preferential induction of Th2- not Th1-type cytokines, as seems to be the case for allograft rejection.[15,46,47] However, as judged by the data above, at least some mechanisms for enhanced xenograft survival may not be critically dependent upon regulation of cytokine synthesis by $CD4^+$ cells. Whether similar observations will hold true for discordant xenografts remains to be explored.

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